REGULATION OF ECDYSTEROID AND VITELLOGENIN LEVELS DURING THE REPRODUCTIVE AND MOLT CYCLES OF FEMALE DUNGENESS CRAB, *CANCER MAGISTER*

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December 15, 2005

Date

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A

THESIS

Presented to the Faculty
of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

By

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Fairbanks, Alaska

December 2005

Abstract

The reproductive cycle of Dungeness crabs is complicated by the requirement for molting prior to mating. The temporal requirements for molting and ovarian maturation may prohibit an annual reproductive cycle in a proportion of crabs. The goal of this study was to quantify temporal concentrations of circulating ecdysteroids and vitellogenins in female crabs during an annual cycle. Hemolymph in laboratory maintained crabs was sampled to assess physiological state (molting or reproduction). In laboratory crabs, ecdysteroid concentrations were low during intermolt (20.3 ng/ml), increased to maximal levels 15 days before ecdysis (1,886.5 ng/ml), and declined to low concentrations (<90 ng/ml) 5 days before ecdysis. Premolt duration was 150 days, with peak molting in November. Vitellogenin concentrations increased 6-fold during induced (via eyestalk ablation) ovarian maturation over a 90 ± 7.4 day vitellogenic period, and 4-fold for natural ovarian maturation over a 75 to 100 day period. The capability to predict ecdysis 150 days before molt and spawning 100 days before egg extrusion through hemolymph analysis is useful in molt and reproductive assessment of Dungeness crab populations. Additionally, in a preliminary study, reproductive failure and shell-disease were induced by physiological stress due to captive environmental conditions.

Table of Contents

List of Figures

List of Tables

List of Appendices

Acknowledgments

I am grateful to my co-advisors Dr. Shannon Atkinson and Dr. Sherry Tamone for their guidance, support and encouragement. I also appreciate the advice and thorough thesis review from Dr. Tom Shirley. Laboratory assistance and training was graciously provided by Kendall Mashburn, Jesse Dutton, and Jacqueline Mitchell. Field work was made possible through the support of Captain Peter Ord and the crew of F/V Williwaw and dive support from Chris Siddon and Elizabeth Calvert. Captive husbandry was made possible by the assistance of the Alaska SeaLife Center aquarium department comprised of: Richard Hocking, Jared Guthridge, Mark Kansteiner, Deanna Trobaugh, and Ed DeCastro. Funding for was provided by the University of Alaska Fairbanks Foundation, the Alaska SeaLife Center, and the Biomedical Research Infrastructure Network (BRIN). As the primary author, I collected all animals and hemolymph samples, provided animal husbandry care, and performed all laboratory analysis, data analysis and thesis preparation. The graduate advisory committee provided scientific and logistical guidance throughout this study.

CHAPTER 1: GENERAL INTRODUCTION

Dungeness crab (*Cancer magister* Dana 1852) inhabit a wide range of coastal waters from Magdalena Bay, Mexico north to Prince William Sound, Alaska and west to the Pribilof Islands in the Bering Sea (Jensen and Armstrong, 1987). In Alaskan waters, Dungeness crab predominantly inhabit protected inshore habitats including bays, fjords and estuaries. Reproductively mature Dungeness crab in Alaska exhibit seasonal movement from deeper waters (>40 m) in winter to shallower waters (<25 m) during spring and summer for egg hatching and subsequent mating, however some ovigerous females remain in shallow waters through the winter (Stone and O'Clair, 2001). From the time of egg extrusion (Sept) until hatching (May/June) ovigerous female aggregations are found on sandy substrates at various depths, commonly less < 10 m (Scheding *et al.*, 2001).

From central California to Kodiak, Dungeness crab populations support significant commercial and recreational fisheries with an exvessel value of $5.4 million in Alaskan waters for the 2003 commercial harvest (Alaska Department of Fish and Game, 2003 Preliminary Alaska Commercial Shellfish Catches & Estimated Exvessel Values, Commercial Fisheries). Crab fisheries regulations are specific to each state, in Alaska the harvest of mature male crabs with a minimum carapace width (CW) of 165 mm, measured immediately anterior to the tenth anterolateral spines, is permitted. Canadian fisheries allow the harvest of mature male and female Dungeness crab with a minimum CW of 165 mm with spine widths included. To effectively manage this important crab

species, thoroughly understanding the reproductive processes and life history of populations throughout the entire range is necessary.

The reproductive cycle of Dungeness crab includes egg maturation, fertilization, egg extrusion, egg incubation (brooding) and larval hatching. Dungeness crab embryos hatch from an egg mass that is attached to maternal pleopods. During the brooding period, developing embryos within egg cases are carried in an open clutch exposed to physical, chemical and biological factors that may cause brood mortality. Physical contact between the egg mass and substrate as the female moves and forages may cause mechanical damage and mortality to 4.0-6.0% of the embryos (Shields, 1991). Chemical agents that may cause additional embryonic mortality in Dungeness crab include chemical pollutants such as chlorine (Horne *et al.*, 1983), pesticides (Buchanan *et al.*, 1970) and inorganic solutes leached from logs at log transfer sites in Alaska (O'Clair and Freese, 1988). Biological predators and parasites such as bacteria, fungi, nemertean worms and amphipods also result in embryonic mortality (Shields, 1991). The rate of embryonic development and larval growth is reduced in Alaskan waters due to colder water temperatures (Wild, 1983; Shirley *et al.*, 1987; Hankin *et al.*, 1997). Hatching occurs from April through August, with the greatest hatching occurring in May and June in Alaska (Shirley *et al.*, 1987). Variability in hatching dates and larval development is also apparent due to temperature differences between outer-coast and inland waters (Sulkin and McKeen, 1996) or perhaps as a result of hatching cues not yet identified.

The larval stages of Dungeness crab are planktonic for three to four months and include a prezoea (which last only 30 to 60 minutes), five zoeal stages and a megalopa

stage. Settlement out of the water column to the benthos is observed beginning in August (Shirley *et al.*, 1987), but most megalopae in southeastern Alaska settle in September and October (G. Eckert, UAS, personal communication). During the first year following settlement, juveniles are estimated to molt six to nine times and two to five times the second year (Wainwright and Armstrong, 1993).

Mating occurs when a hard-shelled male embraces a female preparing to molt. The embrace may last seven to eight days in either a sternum-to-sternum position or sternum-to-carapace position. Immediately prior to molting the female signals to the male with increased antennal and chelae movement, and the male responds by assisting the female in egress from the exuvium (Snow and Nielson, 1966). Probably chemical cues are important in the communication between male and female crab involved in mating. Copulation occurs in a sternum-to-sternum position while the female is in a soft-shell state (Snow and Nielson, 1966). Females are receptive to mating for approximately 10 days after molting and are commonly polygynous (Jensen *et al.*, 1996). After mating, males have been observed to embrace, stand over, or remain near a female for up to two days while the female's exoskeleton sufficiently hardens for protection (Snow and Nielson, 1966). Complete hardening of the exoskeleton can take as long as three months (Shields, 1991). Females become sexually mature at 90 -- 100 mm CW usually in their second year (Hankin *et al.*, 1989). Females may mate when CW is 69 mm, but do not extrude eggs until CW is approximately 105 mm. Physiological sexual maturity is attained in males at an approximate CW of 95 mm (Shields, 1991), however no male has been observed mating at less than 125 mm CW (T. Shirley, personal communication). In

southeastern Alaska, the primary mating period is believed to occur between June and November, however animals have been observed in pre-copulatory embraces in all months except February (Stone and O'Clair, 2001; Swiney and Shirley, 2001). Differing reproductive classes account for the broad molting season, with smaller (<118 mm) females molting between August and April and larger females molting between May and December (Stone and O'Clair, 2001; Swiney and Shirley, 2001).

Sperm is transferred to the female in a spermatophore which is broken during intercourse releasing spermatozoa into the oviducts which lead to the internal spermathecae (Shotton, 1973). A sperm plug seals the spermatozoa in the spermathecae and may prevent mixing of spermatozoa from subsequent mating by other males. Spermatozoa are retained across molts (Shirley and McNutt, 1989) and can be stored in the paired spermathecae for at least 2.5 years, fertilizing eggs as they pass by the spermathecae during extrusion (Hankin *et al.*, 1989; Jensen *et al.*, 1996). The storage of sperm allows some females to skip-molt and extrude eggs without annual molting or mating (Ebert *et al.*, 1983).

Prior to egg extrusion, the female burrows into a soft sand or mud substrate to create a mold or protected chamber between the substrate and her abdomen (Shields, 1991). After oviposition of the egg mass to the abdominal pleopods, brooding occurs for approximately nine months until hatching (Jaffe *et al.*, 1987). In the absence of sediment, (e.g. in laboratory tanks) the eggs do not adhere to the setae and a clutch is not formed. In a laboratory study, extrusion of eggs in Alaskan females occurred between September and November (Swiney and Shirley, 2001). The range of fecundity per brood for

Dungeness crab is between 500,000 to 1,500,000 eggs, and is strongly correlated to the size of the female (Wild, 1983; Hankin *et al.*, 1985; Orensanz and Gallucci, 1988). Dungeness crab typically exhibit three to four adult instars and have an average life span of eight years (Shields, 1991). However, based on skip-molting observations, the maximum ages are probably considerably higher than 8 years; 10 to 12 years is likely in the absence of commercial fisheries (T. Shirley, UAF, personal communication).

Dungeness crab in Californian waters undergo an annual reproductive cycle and continue ovarian development soon after egg extrusion during the brooding period (Wild, 1983). However, in Alaskan waters an unknown proportion of female crabs do not reproduce annually. This may be due to energetic constraints resulting from lower feeding and foraging behavior while brooding eggs (Schultz and Shirley, 1997) and the short interval (3-4 months) between hatching and egg extrusion. The three month period between larval hatching (May/June) and peak egg extrusion (Sept) may be insufficient for molting and ovarian maturation, thus prohibiting annual reproduction for some female crabs (Swiney and Shirley, 2001).

Laboratory studies have demonstrated that Alaska Dungeness crab may or may not reproduce in successive years; almost all larger females did not reproduce in successive years (Swiney and Shirley, 2001). Furthermore, the gonadosomatic index (GSI) only began to increase after hatching (May through July) with peak GSI in September, prior to extrusion (Swiney and Shirley, 2001). Previous field studies in southeastern Alaska, found only 67% of mature females were ovigerous in April 1982 and May 1983, a time when most females should be ovigerous based on an annual reproductive cycle (O'Clair

and Freese, 1988). More recent field data collected in southeastern Alaska during April 1992-1997, found that 85% of females collected in pots and 64% collected on dive transects were nonovigerous (Swiney *et al.*, 2003). A possible explanation is that older females do not molt or extrude eggs annually, and may produce multiple clutches without molting through the use of stored sperm, or enter a senescent period (Hankin *et al.*, 1985). Further evidence using tags, designed to remain attached through a molt, confirmed that an unknown, but large percentage of Alaskan Dungeness crab females did not extrude eggs annually, and skipped at least one annual cycle (Swiney *et al.*, 2003). Determining precise estimates of the percentages of females with and without eggs is difficult because of sampling biases (Taggart *et al.*, 2004).

Female crabs that exhibit annual egg extrusion must undergo ovarian development coincident with molting which requires significant energy resources. These demands coupled with the low water temperatures, slow egg development and decreased foraging in Alaskan waters may affect molting and reproductive periodicity. Field data from April 1992-98 revealed that 45% of nonovigerous crabs were new-shelled compared to 6% of ovigerous crabs, indicating that females that molted the previous summer and fall are less likely to extrude eggs that year. Likewise, 86% of ovigerous crabs were old and very-old shelled compared to 54% of nonovigerous (Swiney *et al.*, 2003). Additionally, only 19% of females recovered in an annual tagging study had molted, verifying that not all mature females molt annually (Swiney *et al.*, 2003). The probability of molt for Dungeness crab in northern California approached 0% in crabs larger than 155 mm CW, and near 100% for crabs less than 130-135 mm CW (Hankin and Xue, 1996). Similarly, a previous

tagging study (Table 1) conducted from 1965-1969 in southeastern Alaska reported a decrease in molting probability with an increase in size (Koeneman, 1985).

These field and laboratory studies indicate that Alaskan female Dungeness crab may undergo any of the following growth and reproduction strategies:

1. Molt and mate annually, with great temporal variation.
2. Forego an annual molt and extrude eggs using stored sperm.
3. Molt in late summer or fall and forego extrusion until the following year.

DUNGENESS CRAB REPRODUCTIVE PHYSIOLOGY

The physiological processes of reproduction and growth are hormonally coordinated and regulated. Hormones are synthesized by endocrine glands and secreted into the hemolymph and circulated to target cells where the hormones influence cell physiology (Reddy and Ramamurthi, 1999; Subramoniam, 2000). Manipulation of endocrine glands and suspected endocrine tissues has been successfully used to identify hormones, locate sites of hormone synthesis and elicit physiological responses. Common techniques include extirpation and reimplantation of tissue, endocrine gland extract injection, bioassay development, hormone purification, and chemical assays such as an ELISA (Chang, 1992). Reviews of hormones in crustacean physiology, specifically reproduction and molting, include Reddy and Ramamurth (1999), Subramoniam (2000), and Meusy and Payen (1988).

The events associated with molting are stimulated directly by the presence of ecdysteroids, commonly called molting hormones, circulating in the hemolymph.

Molting in decapod crustaceans has been well studied and a thorough description of the hormone driven changes that occur during the molt cycle published (Skinner, 1985). Concentrations of ecdysteroids circulating in hemolymph are low during inter-molt, increase significantly during pre-molt and decline drastically just before molting (Chang and O'Connor, 1978).

Ecdysteroids are a group of polyhydroxylated ketosteroids that are present in all arthropods and function to control ecdysis, or molting. In crustaceans, the ecdysial glands known as the Y-organs are responsible for ecdysteroid synthesis (Chang, 1985). Synthesized ecdysone is released into the hemolymph and converted into active 20-hydroxyecdysone (20-HE) by peripheral tissues and promotes the physiological changes associated with molting (Chang *et al.*, 1976). *Cancer antennarius* has additional ecdysteroids also released by the Y-organs, 3-dehydroxyecdysone and 25-deoxyecdysone, a precursor for active ponasterone A (Spaziani *et al.*, 1989). Ponasterone A is the main serum ecdysone present during pre-molt in *Carcinus maenas* and the land crab *Gecarcinus lateralis,* which suggests a molt control function (McCarthy, 1982; Lachaise and Lafont, 1984).

The synthesis and release of ecdysteroids is under inhibitory control by the molt-inhibiting hormone (MIH), a neuropeptide produced by the X-organ sinus gland complex in the eyestalk (Soumoff and O'Connor, 1982), and stimulatory control by methyl farnesoate (MF), a sespiterpenoid compound which is produced in the mandibular organs (Borst *et al.*, 1987; Tamone and Chang, 1993). Dungeness crab Y-organs incubated in

the presence of various concentrations of MF produced significantly higher amounts of ecdysteroids which demonstrates direct control over molting (Tamone and Chang, 1993).

Numerous studies have reported that ecdysteroids do not play a specific stimulatory role in vitellogenesis (for a thorough review, see Meusy and Payen, 1988). In Dungeness crab the relationship between ecdysteroids and vitellogenesis is unknown, however the presence of ecdysone, 20-HE and Ponasterone A in the ovary and embryos has been confirmed (Okazaki and Chang, 1991).

Crustacean embryos receive nutrients and proteins necessary for development from the egg yolk. The process of yolk accumulation by the oocytes, known as vitellogenesis, begins with intra-oocyte vitellin (Vn) synthesis and the internalization of the extra-oocyte precursor to Vn known as vitellogenin (Vg) circulating in the hemolymph. Vn is the major egg yolk protein, which is electrophoretically and immunologically identical to Vg; both are lipo-glyco-carotenoproteins (Adiyodi, 1968; Spaziani *et al.*, 1986; Meusy and Payen, 1988). The location of Vg synthesis is still uncertain, but the hepatopancreas, adipose tissue and ovaries may be the major sites (Paulus and Laufer, 1987; Yano and Chinzei, 1987). Extra-ovarian Vg is endocytotically sequestered by the oocytes and subsequently named vitellin or lipovitellin. The presence of Vn in oocytes is easily determined by the bright color of carotenoid pigments that are linked to Vg.

A widely accepted model for vitellogenesis involves multiple hormones that coordinate the synthesis and uptake of vitellogenins. Vitellogenesis-inhibiting hormone (VIH) is the primary regulating hormone, also referred to as gonad-inhibiting hormone and ovary-inhibiting hormone. VIH is synthesized in the X organ-sinus gland complex

located in the eyestalk of Dungeness crab (Meusy and Payen, 1988). The X organ is within the medulla terminalis of the optic lobes and is the source of VIH and other neurohormones such as MIH. The sinus gland complex is a neurohemal organ that stores and releases VIH directly into the hemolymph where it circulates to specific target tissues (Hodge and Chapman, 1958; Fingerman and Aoto, 1959; Bunt and Ashby, 1967). Removal of the X organ-sinus gland complex through ablation of the eyestalks results in the onset of vitellogenesis and molting (Quackenbush, 1994). Unilateral ablation is the common practice by shrimp farmers since bilateral ablation often results in disadvantageous shortening of life span and various abnormalities of vitellogenesis (Anilkumar and Adiyodi, 1980). Other research has implicated hormones synthesized in the Y-organs (ecdysteroids), mandibular organs (MF) and thoracic ganglion in inhibitory and stimulatory reproductive regulation (Meusy and Payen, 1988).

RESEARCH PROJECT OBJECTIVE

The goal of this research was to elucidate the physiological changes associated with the timing of reproduction and molting in Alaskan Dungeness crab through hemolymph sampling and quantification of reproductive proteins and hormones regulating these processes. The first objective was to determine the temporal changes in hemolymph ecdysteroid concentrations during early premolt, premolt, and ecdysis in Dungeness crab. Ecdysteroids were quantified in longitudinal samples from captive crabs and single samples from a cross-section of wild crabs using an enzyme-linked immunosorbent assay (ELISA).

The second objective was to determine the temporal requirements for vitellogenesis and periodicity in mature female crabs. Hemolymph vitellogenin concentrations were measured using gel electrophoresis. These methods for assessing the growth and reproductive status in Dungeness crab greatly advance our ability to understand the processes of growth and reproduction of Dungeness crab in Alaskan waters and enable us to more effectively monitor and manage this commercially important Alaskan species.

Table 1.1. Molt frequency of 179 recaptured female Dungeness crab (*Cancer magister*) in mm of carapace width (from Koeneman, 1985).

Size Class (carapace width in mm)	% Molted Prior to Recapture	% Recaptured After 1 Year Without Molting	% Recaptured After 2 Years Without Molting
130-134	92	8	-
134-139	71	29	-
140-144	26	74	-
145-149	17	83	-
150-154	3	94	3
155-159	-	87	13
160-164	-	80	20
165-169	-	100	-
170-174	-	100	-

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CHAPTER 2: CIRCULATING ECDYSTEROID CONCENTRATIONS IN ALASKAN DUNGENESS CRAB (*Cancer magister*)

ABSTRACT

The currently accepted reproductive cycle of Alaskan female Dungeness crab (*Cancer magister*) is that females molt and mate during summer and fall, extrude eggs in the fall, and incubate eggs through the winter until larval hatching in May and June. In this cycle, molting and mating are coincident with ovarian maturation. In this study, we quantified circulating concentrations of hemolymph ecdysteroids (molting hormones) in laboratory and field sampled crabs using an enzyme-linked immunosorbent assay (ELISA), for molt status assessment of individuals. Captive female crabs from southeastern Alaska (mean CW=134.8 mm; n=48) had a typical crustacean molt cycle profile of circulating ecdysteroids. Concentrations of ecdysteroids were low during intermolt (20.3 ± 0.7 ng/ml), maximal during premolt (1,886.5 ± 186.2 ng/ml) 15 days before ecdysis and precipitously declined to low levels (<90 ng/ml) 5 days prior to ecdysis. The duration of premolt was 150 days. Crabs held in captivity either molted (67%), extruded eggs (7%), or demonstrated no reproductive or molting activity (26%). Peak molting occurred in November for crabs held in the laboratory. Most (98%) of the female crabs sampled (n=579) in Port Frederick, Alaska during the expected premolt period (May-July), had intermolt ecdysteroid concentrations. The capability to predict ecdysis 150 days prior to ecdysis by measuring ecdysteroid concentrations provides a useful tool to assess the molt status and timing of ecdysis in Alaskan Dungeness crab populations.

*Prepared for and accepted by the Journal of Crustacean Biology. Thomton, Tamone, and Atkinson, 2006.

INTRODUCTION

Dungeness crab (*Cancer magister* Dana 1852) inhabit coastal waters from Magdalena Bay, Mexico to the Pribilof Islands, Alaska (Jensen and Armstrong, 1987). Annual growth and reproductive cycles vary temporally throughout this range; mating occurs between hard shelled males and newly molted soft shelled females (MacKay, 1943; Butler, 1960; Snow and Nielson, 1966). Molting frequency decreases during development, with juveniles molting 6 to 9 times during their first year and 2 to 5 five times during their second year (Wainwright and Armstrong, 1993). Sexual maturity is attained at 90--100 mm carapace width (CW) followed by annual molting and mating events (Hankin *et al.*, 1989). In Californian waters, molting probabilities are near 1 for females less than 140 mm CW and decreases to near zero for females larger than 155 mm (Hankin and Xue, 1996). Similarly in Alaskan waters, adult female crabs molt annually until reaching a CW of 130--135 mm followed by a decrease in molting probability to near zero when CW is greater than 150 mm (Lehman and Osborn, 1970; Koeneman, 1985). Tag and recovery studies in Glacier Bay, Alaska confirm non-annual molting by 81% of recaptured adult females; larger females molted every other year (Swiney *et al.*, 2003).

In Alaskan waters, the primary molting and mating period is thought to occur from June through November, however animals have been observed in pre-copulatory embraces in all months except February (Stone and O'Clair, 2001; Swiney and Shirley, 2001). This large variation in the timing of molting could be explained by differing reproductive classes, with smaller (<118 mm) females molting between August and April

and larger females molting between May and December (Stone and O'Clair, 2001; Swiney and Shirley, 2001). Larger females remain capable of producing consecutive clutches without molting annually by fertilizing oocytes with sperm stored in spermathecae (Hankin *et al.*, 1989; Swiney and Shirley, 2001).

Previous methods employed to assess the probability of molting for individual crabs include rating of shell condition (Somerton and Macintosh, 1983), tag and recovery (Hankin *et al.*, 1989; Swiney *et al.*, 2003), observations of setal morphogenesis (Lyle and MacDonald, 1983) and quantification of molting hormones (ecdysteroids) circulating in crab hemolymph (Chang and Bruce, 1980; Tamone *et al.*, 2005). The latter method of hormone analysis has been used to characterize molting events in many crustaceans (Subramoniam, 2000), however has not been used in premolt determination for Dungeness crab.

Ecdysteroids are a group of polyhydroxylated ketosteroids that are present in all arthropods and function to regulate growth (Hampshire and Horn, 1966). Ecdysone is synthesized in the Y-organs, released into the hemolymph and converted into active 20-hydroxyecdysone (20-HE) by peripheral tissues (Chang and O'Connor, 1977). Ecdysone promotes the physiological changes associated with molting. The synthesis and release of ecdysteroids is under inhibitory control by the neuropeptide molt-inhibiting hormone (MIH), which is produced by the X-organ sinus gland complex in the eyestalks (Soumoff and O'Connor, 1982), and stimulatory control by methyl farnesoate (MF), a sespiterpenoid compound which is produced in the mandibular organs (Borst *et al.*, 1987; Tamone and Chang, 1993). Concentrations of ecdysteroids circulating in hemolymph are

typically low during intermolt, increase significantly during premolt and decline drastically just before molting (Chang and Bruce, 1980).

The temporal and physiological connection between molting and reproduction in Dungeness crabs is unclear. While females must be in a soft-shelled condition to mate, the storage of sperm enables fertilization of a second clutch of oocytes to occur without molting and mating again. The role of ecdysteroids in crustaceans is primarily to control molting, however ecdysteroids are also sequestered in the ovary during vitellogenesis and embryogenesis implying an additional role during reproduction. Ecdysone, 20-hydroxyecdysone, and ponasterone A (free and conjugated ecdysteroids) are present in Dungeness crab embryos (Okazaki and Chang, 1991), and may serve as morphogenetic hormones controlling development or as an elimination pathway, although the specific function is unclear (Subramoniam, 2000).

This paper presents results on circulating ecdysteroid concentrations, measured via an enzyme-linked immunosorbent assay (ELISA) that was validated for Dungeness crabs, during intermolt and premolt periods. This information provides a better understanding of the physiological process of molting as well as probability, timing, and frequency of annual growth cycles for female Dungeness crabs in Alaskan waters, the northern range limits for this species.

MATERIALS AND METHODS

Adult female Dungeness crabs were collected in commercial crab pots from Port Frederick, in southeastern Alaska (lat. 58.2°N, long. 135.4°W) in July, 2003 and May,

June, and July, 2004. Crab pots were set at 5 to 25 m depths with 24 hour soak times. During the May, 2004 collection, SCUBA divers collected all female crabs encountered along transects in the vicinity of the pots at depths between 4 and 25 m. Data collected included: CW (excluding the tenth anterolateral spine), abdominal width (notch between 4^{th} and 5^{th} abdominal segments), shell condition (soft shelled, new shelled, old shelled, very old shelled; (Shirley and Shirley, 1988), and pleopod condition (matted or blackened). Additionally, hemolymph (1 ml) was removed from the base of a walking leg from each female. During the May, 2004 collection, 48 female crabs were transported to captive flow-through tanks at the Alaska SeaLife Center in Seward, Alaska (n=29) and the Juneau Center, University of Alaska Fairbanks, Juneau, Alaska (n=19). Holding tanks for all crabs received unfiltered seawater from 100 m depth in Resurrection Bay at the Alaska SeaLife Center and filtered seawater from 30 m depth in Auke Bay at the Juneau Center. Water temperatures in the tanks were equal to ambient bay water; photoperiod varied between indoor and outdoor tanks, however no significant differences in premolt length or molt timing were observed (Appendix 1). Crabs were fed an assortment of Pacific herring (*Clupea pallasii*), capelin (*Mallotus villosus*), squid (*Loligo opalescens*), razor clam (*Siliqua patula*), and krill (*Euphausia superba) ad libitum* twice weekly. Every two weeks, each crab was weighed and hemolymph (0.5 ml) was sampled and stored at -80° C until assayed for ecdysteroids by a specific ELISA.

An induced molting study was initiated in August, 2004 with 5 intermolt females through X-organ removal via bilateral eyestalk ablation; hemolymph (0.5 ml) was sampled from eyestalk ablated crabs weekly.

All hemolymph samples were extracted in 75% methanol and centrifuged (12,000 rpm x 10 min.). The supernatant was evaporated to dryness and reconstituted in assay buffer (AB; 25 mM sodium phosphate, 150 mM sodium chloride, 1 mM EDTA, 0.1% gelatin, pH 7.5) to a final dilution of 1:40. Extraction efficiency of 78% was determined by adding tritiated ecdysone to hemolymph prior to the extraction procedure. Counts were measured using LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Fullerton, CA).

Ecdysteroids were measured using an ecdysteroid ELISA (Kingan, 1989) modified for decapod crustaceans (Tamone *et al.*, 2005). The ELISA was validated for extracted hemolymph samples diluted from 1:20-10,000. ELISA plate (96-well, flat bottomed, Costar) were incubated overnight at 4° C with 90 µl goat-antirabbit serum and then blocked with buffer (AB, 0.002% sodium azide). Wells were washed three times (Dynex Ultrawash Plus Plate Washer, Chantilly, VA) with phosphate buffered saline (PBS: 10 mM sodium phosphate, 150 mM sodium chloride, 0.05% 20-Tween, pH 7.5). Samples (50 µl) of standard 20-HE (Sigma) or crab hemolymph dilutions were incubated with primary antiserum (50 µl; 1:100,000) and horseradish peroxidase conjugated-ecdysone (50 µl; 1:1,000). Plates were incubated overnight at 4° C and later washed in PBS (3 x 2 min.). Color development was initiated by adding tetramethylbenzidine (TMB) peroxidase substrate solution at room temperature (100 µl; KPL). After 15 minutes of agitation on an orbital shaker, phosphoric acid (100 µl, 1.0M) was added to halt color development. Optical density was measured at 450 nm using an automated plate reader (Dynex MRX Revelation Microplate Reader, Chantilly, VA).

Extracted female hemolymph samples were serially diluted and exhibited parallel displacement to the standard curve. Recovery of 20-hydroxyecdysone standard added to pooled female hemolymph (range 16-500 fmol/well) was 96.2% (SD=14.7; CV=15.3%; y=1.008x-2.25; R^2=.99). Precision of inter-assay coefficients of variation for two internal controls were 12.0% and 19.7% (*n*=77 assays). Intra-assay coefficients of variation were < 10%. Sensitivity of the assay was 4 ng/ml (Appendix 2).

All morphometric data and hormone concentrations are reported as mean ± SEM. Data were analyzed using a one-way ANOVA and Tukey Test (SigmaStat 2.03).

RESULTS

Forty-eight female crabs retained for captive repeat sampling had a mean CW of 134.8 ± 1.4 mm, with a range of 113 -- 153 mm, upon capture. The mean abdominal width was 37.1 ± 0.6 mm. Most of the captive crabs had old shells (98%), the remaining were new shelled. At time of capture, 46% were ovigerous, 40% had blackened or matted pleopod setae, and 14% possessed clean pleopods.

Between May and December, 2004, 66.7% of the captive females molted with peak molting occurring in November (Fig. 1). None of the females that molted subsequently extruded eggs; however no males were available for postmolt copulation. 6.7% of the females extruded eggs without molting, and the remaining 26.6% neither molted nor extruded eggs.

Circulating ecdysteroid concentrations in premolt crabs during captivity varied between 4.2 and 1886.5 ng/ml (Fig. 2). Intermolt hemolymph ecdysteroid concentrations

in non-molting females were 20.3 ± 0.7 ng/ml. Early premolt concentrations of 90 ng/ml differed statistically from intermolt values (ANOVA, $P \leq 0.0001$; Tukey's Test, $P < 0.05$) and were detected 150 days before molting. Ecdysteroid concentrations peaked at 1,886.5 ± 186.2 ng/ml 15 days before molting. A precipitous decline to low concentrations (<90 ng/ml) occurred prior to molting.

Females sampled and released in the field during July, 2003 and May, June and July, 2004 had mean CW of 160.6 ± 0.3 mm, ranging from 122 to 188 mm. The mean abdominal width was 47.1 ± 0.2 mm. A majority of the females sampled in Port Frederick in 2003 and 2004 were either old or very-old shelled and had brooded eggs since the previous molt (Table 1). The hemolymph ecdysteroid concentrations for crabs sampled in Port Frederick implies that greater than 97.5% of the crabs sampled were in intermolt (Fig. 3). The mean ecdysteroid concentration in female crabs classified as being in intermolt was 22.9 ± 0.7 ng/ml.

In the 3 months following eyestalk ablation, hemolymph ecdysteroid concentrations remained at intermolt concentrations (mean 21.9 ± 7.4 ng/ml) for 4 of 5 crabs. The 4 crabs with low ecdysteroid concentrations extruded eggs 90 ± 7.4 days post-ablation. The ecdysteroid concentration in the fifth crab was 74.8 ng/ml when ablated, and increased to 2,591 ng/ml 48 days before molting. Ecdysteroid concentrations subsequently declined gradually, rather than precipitously, until ecdysis occurred 167 days after eyestalk ablation.

DISCUSSION

Dungeness crab ecdysteroid concentrations followed the same molt cycle profile as demonstrated in other crustaceans. Basal concentrations were low during intermolt, increased 100-fold by late premolt and sharply declined before ecdysis to postmolt concentrations. The duration of premolt was longer than noted from earlier studies with early premolt ecdysteroid concentrations present 150 days before ecdysis. This could be due to slower metabolic processes of crabs near the northern range limits for this species. The duration of premolt in adult female Californian Dungeness crabs is 84 days as measured by setal morphogenesis (Miller and Hankin, 2002). The duration of the Alaskan Dungeness crab premolt is lengthy in comparison to other crustaceans, such as the northeastern Atlantic adult female lobster *Homarus americanus* 50 day premolt (Chang, 1985), the salt marsh and mud flat fiddler crab *Uca pugilator* 22 day premolt (Hopkins, 1983), and the coastal southeastern United States blue crab *Callinectes sapidus* 12 day premolt (Soumoff and Skinner, 1983).

We found that 66.7% of the smaller (134.7 mm CW) captive females molted during an annual cycle, which supports the findings that not all female Dungeness crabs molt and reproduce annually (Swiney *et al.*, 2003). These smaller females were one or two molt increments smaller than crabs sampled in the field during this study (160.6 mm CW), and thus had a higher probability of molting (Lehman and Osborn, 1970). In these smaller captive crabs, ecdysis occurred in autumn (Fig. 1), near or after typical egg extrusion has been observed in Alaska Dungeness crabs. Furthermore, vitellogenesis was absent or incomplete (as determined by dissection) in all ecdysial crabs, leaving little time for

oocyte maturation and egg extrusion. Consequently autumnal ecdysis precluded ovarian maturation for two-thirds of these adult female crabs. Based on the physical condition of the pleopods at time of capture, 86% of the females had brooded eggs since their last molt. Additionally, at time of capture 98% were classified as old shelled and therefore had not completed the annual molt before the date of capture. Thus, the probability of annual molting events was high since very old shelled females are uncommon in this size range (Lehman and Osborn, 1970). These data strongly support a non-annual growth and reproductive strategy.

As expected, the likelihood of molting for the larger field sampled crabs was extremely low, with less than 3% of large females in premolt in 2003 (161.4 mm mean CW), and 2% in premolt in 2004 (159.5 mm mean CW). This indicates either a skip-molt strategy by the majority of large adult females, the possibility of senescence, or geographic separation of molting and non-molting animals. Further field sampling of hemolymph ecdysteroids of smaller crabs ranging from 100 to 150 mm CW during the premolt months of May to November is needed to better refine the molting probabilities and our understanding of the life history of adult female Dungeness crabs in Alaskan waters.

Eyestalk ablation of non-ovigerous female crabs during intermolt suggested that molt induction through eyestalk ablation is dependent upon the reproductive state of the crab. Only one of the five mature female crabs entered premolt, while the other four extruded eggs and maintained low intermolt ecdysteroid levels. Of interest is the 167 day period between eyestalk ablation and molting for the single induced molting crab, which is

similar to the 150 day premolt period observed in the naturally molting captive crabs. The removal of the X-organ and eradication of molt inhibiting hormone (MIH) typically induces premolt, however other factors such as crustacean hyperglycemic hormone, methyl farnesoate (MF), xanthurenic acid, calcium, and vitellogenesis inhibiting hormone have been implicated in regulation of ecdysis and reproduction (Borst *et al.*, 1987; Mattson and Spanziani, 1987; Naya *et al.*, 1989; Webster, 1993). For example, the sesquiterpenoid hormone, MF, which is synthesized in the mandibular organ and under direct control of a sinus gland neuropeptide (Borst *et al.*, 1988), has regulatory control of ecdysteroid synthesis in the Y-organ (Tamone and Chang, 1993) and its increase upon eyestalk ablation may account for induction of ecdysteroids in ablated animals (Chang and Bruce, 1980). However, the regulatory relationship between MIH, MF and ecdysteroids remains uncertain. Growth and reproduction are competitive physiological processes in the utilization of energy reserves, thus the reproductive state of a female crab may influence the molt cycle as reported for the American lobster *Homarus americanus,* where the molt interval was lengthened 130 days for females undergoing reproductive vitellogenesis (Chang, 1985). Under normal conditions in the American lobster, if a female enters premolt (D_1) while undergoing vitellogenesis, molting takes precedence and yolk proteins are resorbed (Byard and Aiken, 1984). The same is true of the brachyuran crab, *Metapograpsus messor* (Sudha and Anilkumar, 1996).

Our findings further accentuate the complexity and variability of the annual growth and reproductive cycles for Dungeness crabs in Alaskan waters. However, the capability to predict ecdysis 150 days premolt by measuring ecdysteroid concentrations provides a

useful tool for further refinement in our understanding of the transition from annual to non-annual growth and reproduction between smaller (100 mm CW) females to the larger (160 mm CW) highly fecund mature female crabs. This technique could also be useful in assessing molting probabilities in commercially important male Dungeness crabs.

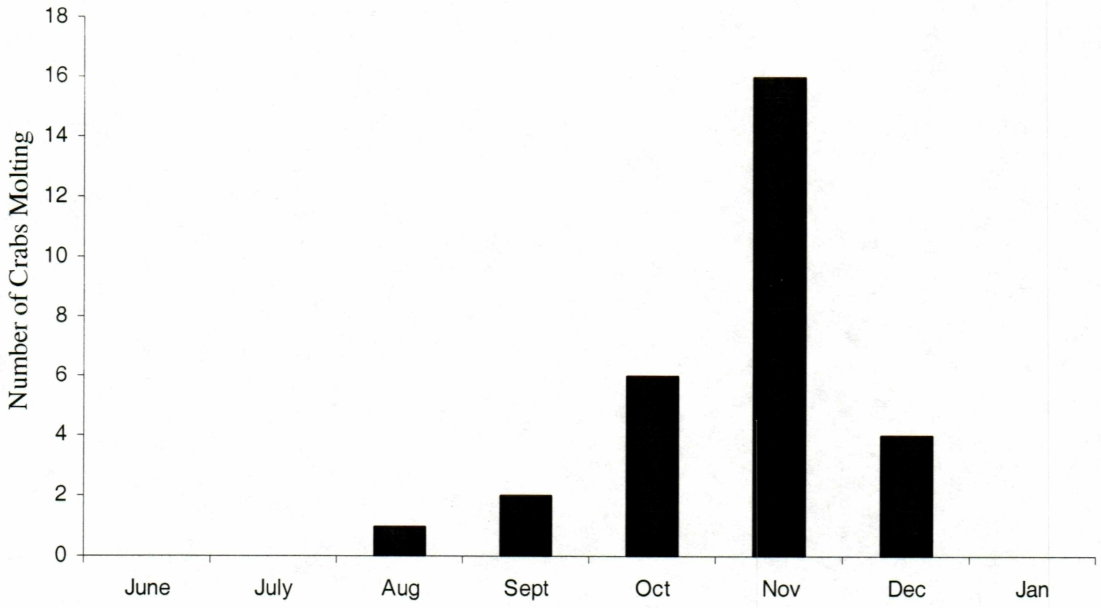

Fig. 2.1. Molting activity of female Dungeness crabs (n=48) maintained in captivity. Crabs were collected in Port Frederick, Alaska. Peak molting occurred in November.

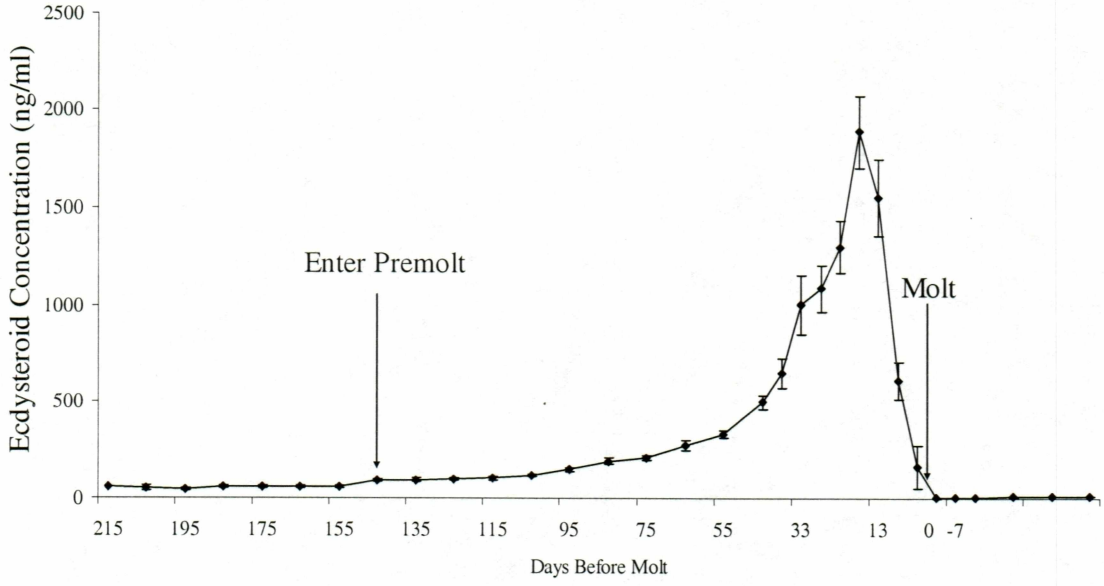

Fig. 2.2. Ecdysteroid concentrations (mean ± SEM) for molting female Dungeness crabs (n=29) maintained in captivity. Crabs (n=48) were collected in Port Frederick, Alaska. Determination of premolt condition based on circulating ecdysteroids permits molt projection 150 days before ecdysis.

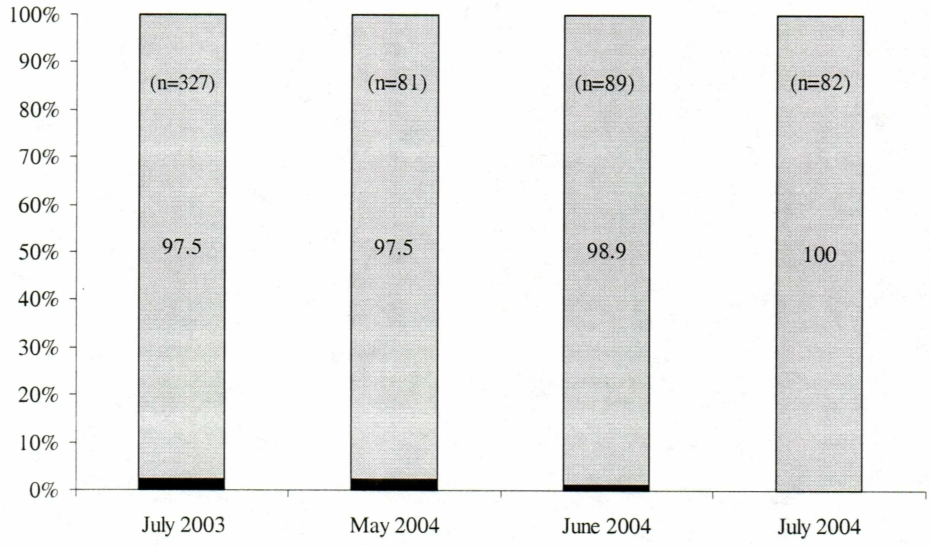

Fig. 2.3. Percentage of female Dungeness crabs sampled in Port Frederick, Alaska in intermolt (shaded bars), or early premolt (solid bars), based on ecdysteroid concentrations circulating in hemolymph, during the annual premolt period.

Table 2.1. Shell and pleopod condition of female Dungeness crab (*Cancer magister* Dana 1852) in Port Frederick, Alaska.

	Shell Condition				Pleopod Condition		
	Soft	New	Old	Very Old	Blackened	Eggs	Clean
July 2003 (*n*=327)	1.2%	35.3%	45.8%	17.7%	55.0%	--	45.0%
May 2004 (*n*=81)	--	21.0%	74.1%	4.9%	20.0%	26.3%	54.0%
June 2004 (*n*=89)	--	15.6%	73.3%	11.1%	60.0%	18.9%	21.1%
July 2004 (*n*=82)	--	24.7%	71.8%	3.5%	64.7%	2.4%	32.9%

ACKNOWLEDGEMENTS

This project was supported by University of Alaska Foundation and by NIH Grant Number RR-16466-01 from the RFIP of the National Center for Research Resources. The conjugated ecdysone and primary antiserum were purchased from Dr. Timothy Kingan (UC Riverside). We thank the Alaska SeaLife Center for the use of facilities and the following people for their assistance with crab collections: Jacqueline Mitchell, Chris Siddon, Elizabeth Calvert, Matt Richards and Captain Peter Ord and the crew of F/V Williwaw. We appreciate the laboratory assistance provided Jessica Dutton and Kendall Mashburn. We thank Richard Hocking, Jared Guthridge, Mark Kansteiner, Deanna Trobaugh, and Ed DeCastro at the Alaska SeaLife Center for crab husbandry and hemolymph sample collection. We especially thank Dr. Thomas Shirley for project guidance and editorial support.

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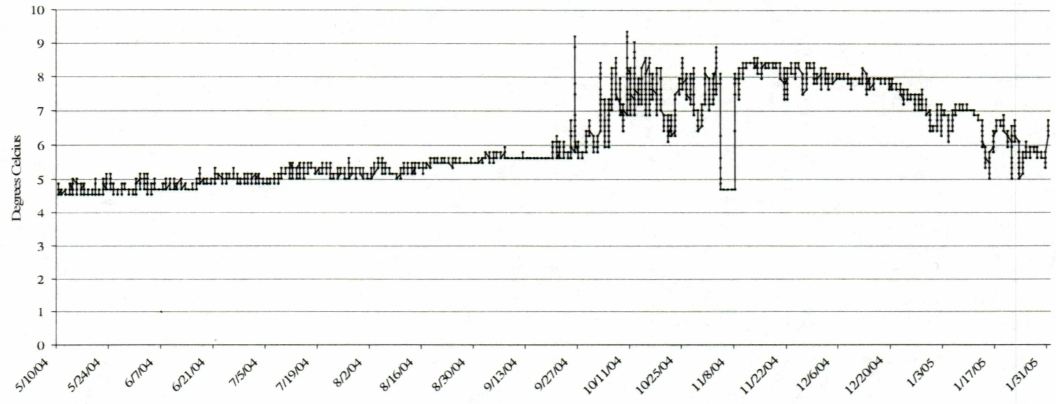
Appendix 1.

ENVIRONMENTAL CONDITIONS

Temperature Logs.--Stowaway Tidbit Temperature Loggers (Onset Computer Corporation, Bourne, MA) recorded tank temperature hourly in each of the three captive tanks at the Alaska SeaLife Center laboratory. Temperatures were consistent between each of the three tanks and with seawater temperatures in Resurrection Bay (Fig. A1.1).

Salinity and Dissolved Oxygen.--Monthly averages at the Alaska SeaLife Center for incoming seawater salinity and dissolved oxygen exhibit negligible variation over the duration of the experiment (Table A1.1).

(a)



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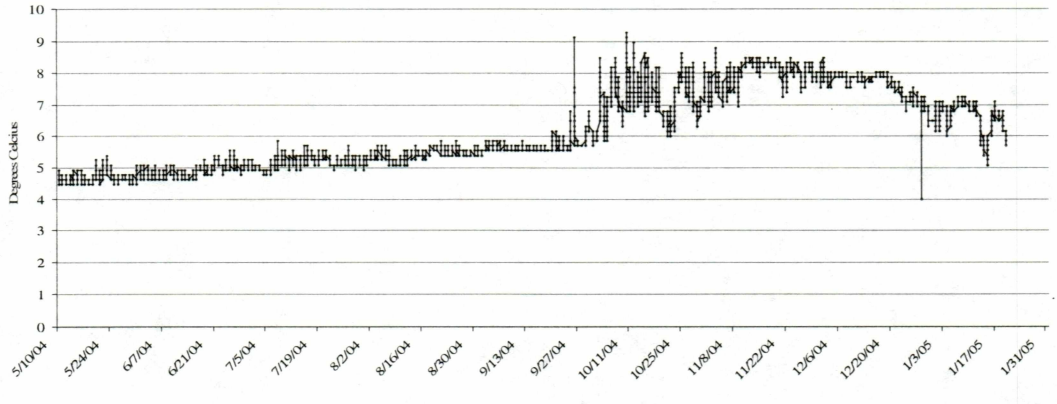


(c)

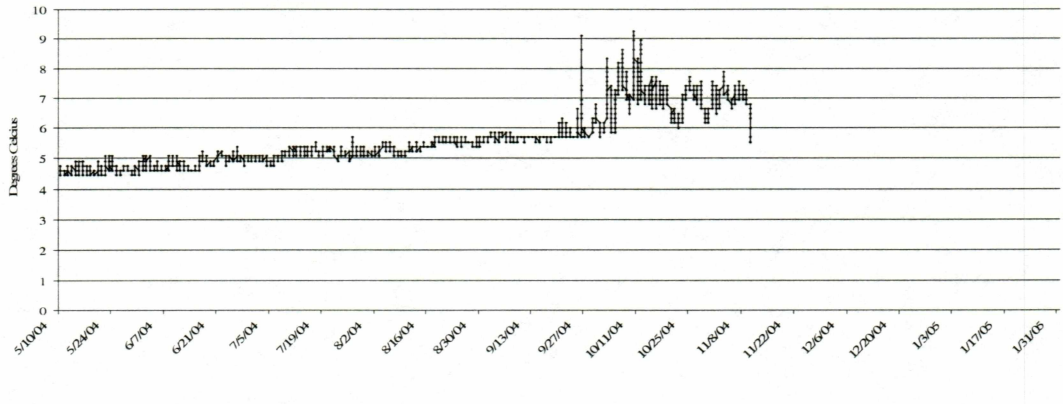


Fig. A1.1. Hourly temperature recordings for each captive Dungeness crab holding tank a) south tank, b) outdoor tank, and c) west tank at the Alaska SeaLife Center, in Seward, Alaska. End dates vary based on tank occupancy.

Table A1.1. Average monthly salinity and dissolved oxygen recordings of incoming seawater from Resurrection Bay to flow-through tanks for the captive Dungeness crab study at the Alaska SeaLife Center, in Seward, Alaska.

Month (2004)	Salinity (PSU)	Dissolved Oxygen (mg/l)	Dissolved Oxygen (%)
May	31.4	10.2	97.0
June	31.5	9.9	95.1
July	31.6	9.0	87.3
August	31.7	9.0	88.5
September	32.0	8.7	86.1
October	32.1	9.0	90.9
November	33.2	8.1	89.6
December	33.6	7.9	83.3

Appendix 2.

ASSAY VALIDATION

Parallelism.--Pooled and extracted female hemolymph samples were serially diluted and exhibited parallel displacement to the standard curve (Fig A2.1).

Hemolymph curve: $y = -0.1482\text{Ln}(x) + 0.3967$; $R^2 = 0.8785$

Standard curve: $y = -0.1606\text{Ln}(x) + 0.9954$; $R^2 = 0.956$

The difference between the slopes is less than 10% (8%), thus validating parallelism for the assay.

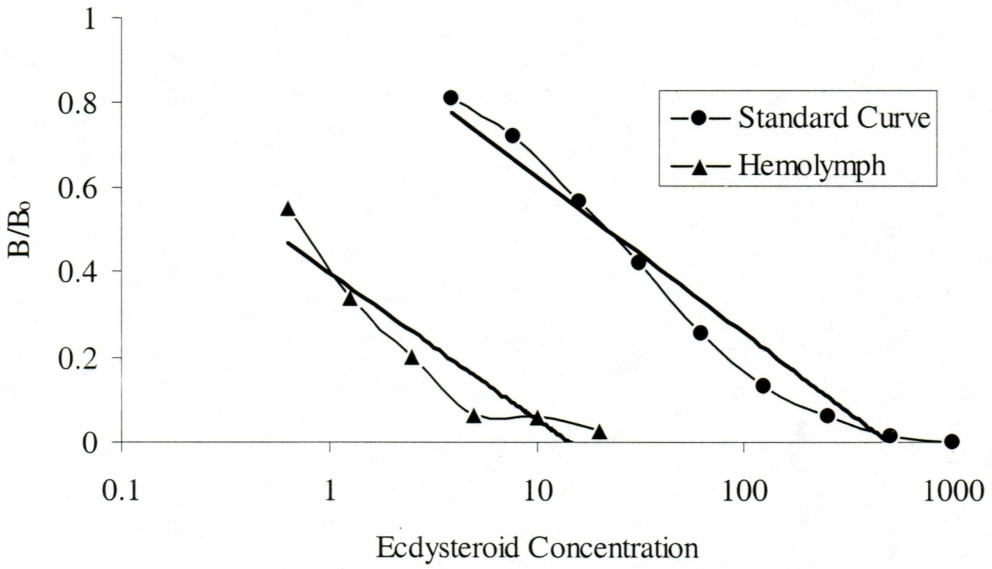
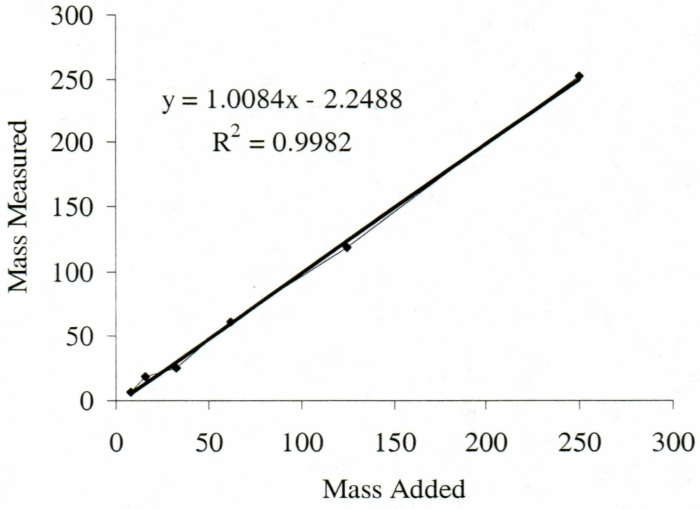


Fig. A2.1. Standard curve and serially diluted extracted hemolymph ecdysteroid concentrations plotted against ratio of optical density (OD) of known dilution (B) and OD of zero ecdysone (B_o).

Recovery.--Recovery of 20-hydroxyecdysone standard added to pooled female hemolymph (range 16-500 fmol/well) was 96.2% (SD=14.7; CV=15.3%; y=1.008x-2.25; R^2=.99) (Fig. A2.2).

(a)



(b)

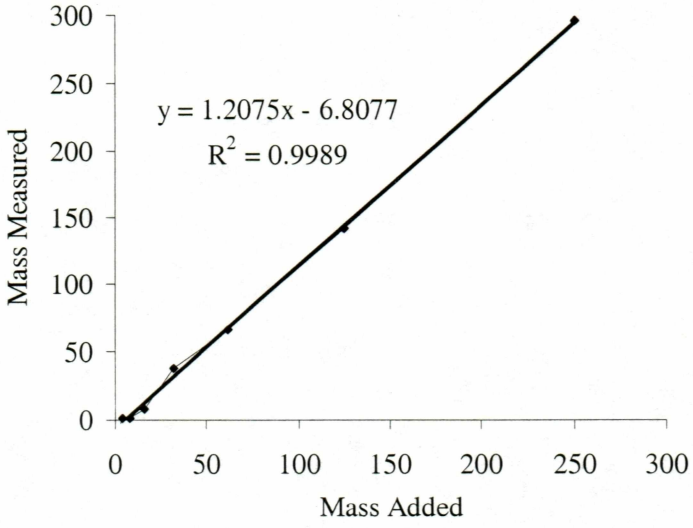


Fig. A2.2. Recovery of known amount of ecdysone standard added to (a) extracted hemolymph and (b) unextracted hemolymph (B). Slope of 1.0 for regression of extracted hemolymph versus slope of 1.2 for unextracted hemolymph.

Assay Sensitivity.--The 4 ng/ml ecdysteroid standard is significantly different from 0 ng/ml standard, thus the assay is sensitive to 4 ng/ml.

Assay Precision.--An internal standard comprised of pooled female hemolymph at two concentrations, highpoint (80% B/B_o) and midpoint (50% B/B_o) on the standard curve, was assayed in duplicate on each plate and resulted in inter-assay coefficients of variation of 12.0% and 19.7% respectively (n=77). All unknown samples were run in duplicate and results accepted when intra-assay coefficient of variation was less than 10%.

Chapter 3: Quantification of circulating vitellogenin in eyestalk ablated and intact female Dungeness crabs *Cancer magister* during a reproductive cycle

ABSTRACT: In Alaska, the temporal and physiological relationship between molting and reproduction in Dungeness crabs *Cancer magister* Dana, 1852, is unclear. Crabs that extrude eggs annually must molt, mate, and undergo ovarian development between June and September. The premolt period requires 150 days in Alaskan crabs and does not overlap with ovarian maturation. The time required to complete vitellogenesis however, is unknown. The goal of this study was to assess vitellogenesis by quantifying circulating vitellogenin (Vg) relative to a non-vitellogenic baseline, during ovarian maturation in induced egg extruding crabs. Vitellogenesis was induced via bilateral eyestalk ablation (EA). Four of 5 EA crabs extruded eggs 90 ± 7.4 days after ablation. Vitellogenin concentrations increased approximately 6-fold during ovarian maturation from low levels (1.2 ± 0.3), to peak levels (6.0 ± 1.1) 25 days prior to egg extrusion, followed by a decrease (2.1 ± 0.5), 5 days prior to egg extrusion. Three of 14 intact captive crabs extruded eggs. Circulating Vg increased from low baseline levels (1.0 ± 0.02) 130 days prior to extrusion, to maximal levels (4.0 ± 1.5) 75 to 100 days before egg extrusion and declined to low levels (1.3 ± 0.2) 10 days before egg extrusion. In EA females, vitellogenesis required at least 90 days. In intact females, circulating Vg was detectable ≥100 days before extrusion, enabling the reproductive assessment of individual crabs 3 months prior to extrusion.

*Prepared for submission to the Marine Ecology Progress Series. Thomton, Tamone, and Atkinson, 2006.

KEY WORDS: Dungeness crab, *Cancer magister,* vitellogenin, vitellin, female-specific protein, reproduction, eyestalk ablation

INTRODUCTION

In decapod Crustacea, the process of yolk accumulation by the oocytes, known as vitellogenesis, begins with intra-oocyte vitellin (Vn) synthesis and the internalization of the extra-oocyte circulating precursor to Vn known as vitellogenin (Vg). During the phase known as secondary vitellogenesis (Dehn et al. 1983), the female-specific protein, Vg, is synthesized and secreted by extraovarian tissues into the hemolymph and endocytotically sequestered by the oocytes. The concentration of Vg in hemolymph during this phase correlates with ovarian development and rapid yolk accumulation by oocytes (Okumura et al. 1992). The molecular mass of crustacean Vg ranges from 260 to 560 kDa, and composed of 2 or more subunits (Meusy 1980, Puppione et al. 1986, Komatsu et al. 1993, Chang et al. 1994). *Cancer antennarius,* a congener to the Dungeness crab (*Cancer magister* Dana 1852), has three peptide subunits of 78 kDa, 107 kDa, and 190 kDa (Puppione et al. 1986). Modification of Vg occurs within the oocytes, and Vg is subsequently referred to as vitellin or lipovitellin. Vitellogenin is the nutritive source of proteins, lipids and carbohydrates for developing embryos. However, Vg and vitellin are electrophoretically and immunologically identical (Lee & Watson 1994); both are lipo-glyco-carotenoproteins (Adiyodi 1968, Spaziani et al. 1986, Meusy & Payen 1988, Pateraki & Stratakis 1997). The source of Vg remains uncertain and varies between species; however the hepatopancreas, adipose tissue and ovaries are the major sites of secretion (Paulus & Laufer 1987, Yano & Chinzei 1987, Fainzilber et al. 1992, Cheng et al. 1999, Tseng et al. 2002). The presence of Vn in oocytes is easily detected by the bright color of carotenoid pigments linked to Vg (Wallace et al. 1967).

The physiological regulation of vitellogenesis involves multiple hormones that coordinate the synthesis and uptake of Vg and has been reviewed (Meusy & Payen 1988, Chang 2001). The X-organ sinus gland is within the medulla terminalis of the optic lobes and is the source of the vitellogenin-inhibiting hormone (VIH; Soyez et al. 1991), the primary hormone that inhibits the synthesis of Vg, and other neurohormones such as the molt-inhibiting hormone (Soumoff & O'Connor 1982). The sinus gland is a neurohemal organ that stores and releases eyestalk neurohormones directly into the hemolymph where they circulate to specific target tissues (Hodge & Chapman 1958, Fingerman & Aoto 1959, Bunt & Ashby 1967). Removal of the X-organ sinus gland complex through ablation of the eyestalks results in the onset of vitellogenesis (reviewed by Quackenbush 1994) and/or molting (reviewed by Chang 1989). The timing of eyestalk ablation in brachyuran crabs, such as the Dungeness crab may determine whether an animal enters premolt or undergoes vitellogenesis (Adiyodi 1988). Other research implicates hormones synthesized in the Y-organs (ecdysteroids), methyl farnesoate (Homola & Chang 1997) and the thoracic ganglion in inhibitory and stimulatory reproductive regulation (for thorough reviews, see Meusy & Payen 1988, Chang 1989).

In Alaska, the temporal and physiological relationship between molting and reproduction in Dungeness crabs is unclear. Female crabs that exhibit annual egg extrusion must molt and undergo ovarian development during the summer months (June-September) which requires significant energy resources. These demands coupled with the colder water temperatures, slow egg development, and decreased foraging in Alaskan waters may affect molting and reproductive periodicity. For example, in southeastern

Alaska an indeterminate percentage of females skip the annual molt for at least one year and extrude viable eggs using sperm stored from previous molt/mate events (Swiney & Shirley 2001, Swiney et al. 2003). Increasing Vg concentrations during vitellogenesis have not been studied in Dungeness crab and the aim of this study was to quantify the temporal change in circulating Vg in EA and intact female crabs during ovarian development. This information will present a better understanding of the process of vitellogenesis in Dungeness crab and provide an effective method to assess the reproductive physiology of female crab populations in Alaskan waters.

MATERIALS AND METHODS

Adult female Dungeness crabs (n=48) were collected in the spring using commercial crab pots and by divers using SCUBA at depths between 4 and 25 m from Port Frederick in southeastern Alaska (lat. 58.2°N, long. 135.4°W). The crabs were transported to captive flow-through tanks at the Alaska SeaLife Center in Seward, Alaska and the Anderson Marine Laboratory in Juneau, Alaska. Holding tanks received filtered seawater from 30 m depth in Auke Bay at the Juneau Center and unfiltered seawater from 100 m depth in Resurrection Bay at the Alaska SeaLife Center. Water temperatures in the tanks were ambient to bay water; photoperiod varied between indoor and outdoor tanks. Crabs were fed an assortment of Pacific herring (*Clupea pallasii*), capelin (*Mallotus villosus*), squid (*Loligo opalescens*), razor clam (*Siliqua patula*), and Antarctic krill *ad libitum* twice weekly. Every 2 weeks for intact crabs and weekly for EA crabs, 0.5 ml of hemolymph was withdrawn from the base of a periopod and stored at -80° C for future assays.

A group non-vitellogenic crabs that were in the intermolt stage of the molt cycles (as determined by measuring circulating ecdysteroids: Thomton et. al. 2005, in review) were EA to induce vitellogenesis. Eyestalks were bilaterally ablated at the base of the eyestalk using fine scissors. Wounds were not cauterized and there was no mortality associated with this procedure. Hemolymph (0.5 ml) was withdrawn from the base of a periopod from all EA crabs weekly and stored at -80° C for future assays. Hemolymph samples were taken before and after egg extrusion.

Total protein in whole hemolymph samples was determined using a total protein assay kit (Biorad, Hercules, CA). Bovine serum albumin served as the standard; absorbance of all samples was determined at 595 nm.

Diluted hemolymph samples containing 25 ug of total protein were prepared for gel electrophoresis by adding an equal volume of sample buffer (Laemmli) and denatured (100° C for 8 minutes). SDS-Polyacrylamide gel electrophoresis was performed using the Mini-PROTEAN® 3 Cell (Biorad) and samples separated using 7.5% precast gels (Biorad, Hercules, CA) at a constant voltage of 200V for 45 minutes with a Tris/glycine buffer system (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). Sequential hemolymph samples from single crab individuals were separated on a single gel. Protein bands in the gels were visualized using the Silver Stain Plus system (Biorad, Hercules, CA).

Silver stained gels were scanned using the Kodak Image Station 440 CF (Eastman Kodak Company, Rochester, NY). The protein band of interest was chosen as a predominant Vg subunit with a molecular mass of 116 kDa. Kodak 1D Image Analysis

Software version 3.6.4 was used to quantify net intensity of the migrated band in each hemolymph sample. The manual region of interest analysis feature was used to quantify the net intensity of Vg bands for relative comparison.

All values are reported as mean relative Vg ± SEM. Relative Vg values were calculated by dividing net intensity of the Vg subunit band at 116 kDa MM by the baseline non-vitellogenic net intensity, specific to each crab. Data were analyzed using a one-way ANOVA and Tukey Test (SigmaStat 2.03) to test for significant differences.

RESULTS

Both intact and EA crabs completed ovarian maturation and extruded eggs in the laboratory. Four out of the 5 EA crabs extruded eggs 90 ± 7.4 days after eyestalk ablation. Vitellogenin concentrations are expressed relative to a non-vitellogenic baseline values and increased after eyestalk ablation. Vitellogenin levels ranged from low baseline values (1.0 ± 0.1), to peak levels (6.0 ± 1.1) 25 days before egg extrusion (Fig. 1). Peak Vg levels decreased (2.1 ± 0.5) 5 days before egg extrusion. Circulating Vg values increased after egg extrusion in the EA group. The increase in circulating Vg between each data point from 90 to 65 days before extrusion was statistically insignificant. There is a significant increase in circulating Vg from 1.0 ± 0.1, prior to ablation to 2.2 ± 0.5, 55 days before extrusion (Fig.1; one-way ANOVA, $P \leq 0.01$; Tukey's Test, $P < 0.05$). Circulating ecdysteroid (molting hormone) concentrations in these four EA crabs remained at intermolt levels (<90 ng/ml) throughout the experiment. Vitellogenin concentrations did not increase in the fifth EA crab; however circulating

ecdysteroids began to rise soon after EA. Ecdysteroids continued to increase until the crab molted 167 days after EA.

Between May, 2004 and February, 2005, 3 of 14 intact non-molting crabs extruded eggs, 2 in the Juneau laboratory and 1 in the Seward laboratory. Vitellogenin concentrations were highest 75 to 100 days before egg extrusion and declined to low non-vitellogenic levels prior to extrusion. The mean relative Vg concentrations for intact egg extruding crabs (n=3), decreased from a relative Vg peak (4.0 ± 1.5), 90 days before extrusion to low levels (1.3 ± 0.2), 10 days before extrusion (Fig. 2). Although the values did not differ statistically, there was a trend toward increasing Vg levels prior to egg extrusion. The ecdysteroid concentrations of the intact egg extruding crabs were consistent with intermolt stages.

The circulating Vg concentrations during vitellogenesis in the two female crabs maintained in the Juneau laboratory appeared to fluctuate synchronously during the 100 days preceding egg extrusion (Fig. 3). Crab B extruded eggs 18 days before Crab A. In non-vitellogenic female crabs, the circulating vitellogenin levels remained low between -0.3 to 1.0 (Fig. 4).

DISCUSSION

The removal of the X-organ, through eyestalk ablation, resulted in the significant onset of vitellogenesis within 35 days for 4 of 5 crabs. In comparison, Vg was present in the hemolymph of the land crab, *Potamon potamios,* 15 to 17 days following EA (Pateraki & Stratakis 2000). A single eyestalk ablated crab remained non-vitellogenic and demonstrated increased circulating ecdysteroids indicative of induced premolt. This

crab molted 167 days post EA. The differing physiological response of these crabs to EA indicates that this procedure can have various outcomes based on the initial physiological state of the crab. It could be assumed that removal of the eyestalks and the associated inhibiting hormones could lead to the induction of both ecdysteroids and vitellogenin, but that was not observed. Eyestalk ablation of intermolt crabs occurred in August, thus vitellogenesis may have been in progress in 4 of the 5 crabs although vitellogenin levels were similar to non-vitellogenic crabs. The fifth molting crab was in intermolt based upon circulating ecdysteroids (74.8 ng/ml) prior to EA, but may have been progressing towards premolt. It appears that crabs in Alaskan waters cannot undergo vitellogenesis and molting simultaneously.

Circulating levels of Vg in the hemolymph of EA and intact extruding Dungeness crabs displayed a pattern similar to other crustacean species (Byard & Aiken 1984, Jasmani et al. 2000, Tsukimura et al. 2000) where maximum Vg concentration is observed well before egg extrusion. This may coincide with the time when maximum accumulation of extra-oocyte Vg begins or may be due to a direct decrease in Vg synthesis by the hepatopancreas. In the EA crabs, a decrease in Vg synthesis by the hepatopancreas, in the absence of VIH, suggests additional regulators of Vg synthesis. This result is analogous to that demonstrated in EA lobsters that show a marked decrease in circulating ecdysteroids prior to molting even in the absence of the molt inhibiting hormone (Chang & Bruce 1980). In this case, it is thought that hormones outside of the X-organ sinus gland complex are responsible for the important decrease in circulating ecdysteroids that initiate ecdysis (Chang 1989).

Vitellogenin levels in EA crabs were elevated above baseline levels for approximately 70 days prior to egg extrusion. The increased levels occurred soon after EA and displayed little variation among the experimental animals. In contrast, Vg levels in intact animals increased 100 days or more before extrusion with great variation among crabs. The variability in the intact extruding crabs might occur in response to unknown vitellogenesis regulating factors, possibly environmental cues such as photoperiod, water temperature or tides. The EA crabs however, responded decisively to the absence of eyestalk hormones with a steady increase of Vg or ecdysteroids and underwent either vitellogenesis or ecdysis.

Of note is the difference in Vg concentration profiles between the intact extruding crabs maintained in different laboratories. The 2 female crabs held in Juneau had synchronously fluctuating Vg concentrations (Fig. 3), despite an 18 day difference in extrusion date. These fluctuations may have been in response to environmental variations (Quackenbush 1994). Crab C, maintained in the Seward laboratory, experienced less Vg variability with a stepped decrease in circulating Vg during the 100 days preceding extrusion, however further experiments with a larger sample size of intact extruding female crabs are needed to better refine this Vg profile.

The data presented in this study demonstrates that the quantification of hemolymph Vg using the techniques described above or through an enzyme-linked immunosorbent assay (ELISA) are effective methods to assess the reproductive status of female crabs. To date there is not a Vg ELISA specific for Dungeness crabs, although this would be a useful tool for measuring reproductive physiology in many economically

important crab species. The duration of vitellogenesis is in excess of 100 days and provides a large sampling window to conduct field surveys and hemolymph collection to determine the reproductive status of female crabs in a given year. The percentage of female Alaskan Dungeness crabs that are vitellogenic, in a given year and in specific locations, could be predicted and included in the life history models which support the management of the Dungeness crab fishery. Future development of a Vg ELISA accompanied by greater sampling of reproductive crabs (≥100 mm carapace width) would significantly enhance our ability to monitor crab stock fecundity and assist in the management of this commercially important species.

ACKNOWLEDGMENTS

This project was supported by University of Alaska Foundation and by NIH Grant Number RR-16466-01 from the RFIP of the National Center for Research Resources. We thank the Alaska SeaLife Center for the use of facilities and the following people for their assistance with crab collections: J. Mitchell, C. Siddon, E. Calvert, M. Richards and Captain P. Ord and the crew of F/V Williwaw. We thank R. Hocking, J. Guthridge, M. Kansteiner, D. Trobaugh, and E. DeCastro at the Alaska SeaLife Center for crab husbandry and hemolymph sample collection. We especially thank Dr. T. Shirley for project guidance and editorial support.

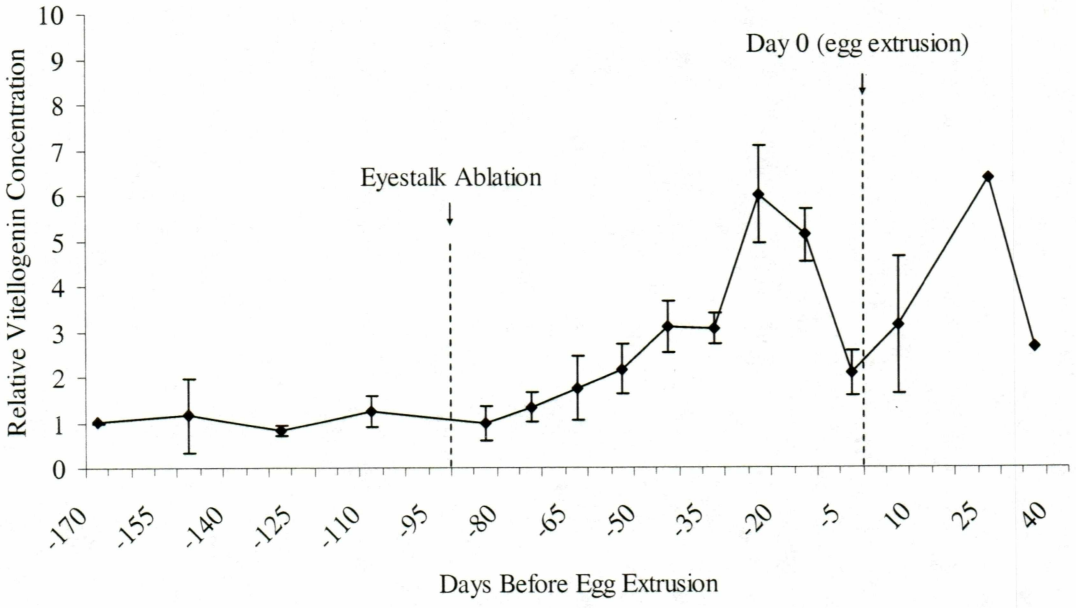


Fig. 3.1. Hemolymph vitellogenin (Vg) levels (relative to non-vitellogenic baseline; mean ± SEM) in eyestalk ablated female Dungeness crabs (n=4). Vg levels increased after ablation to peak levels (6.0 ± 1.1), 25 days before extrusion. Subsequently, circulating Vg decreased until 5 days before extrusion when concentrations began to rise again.

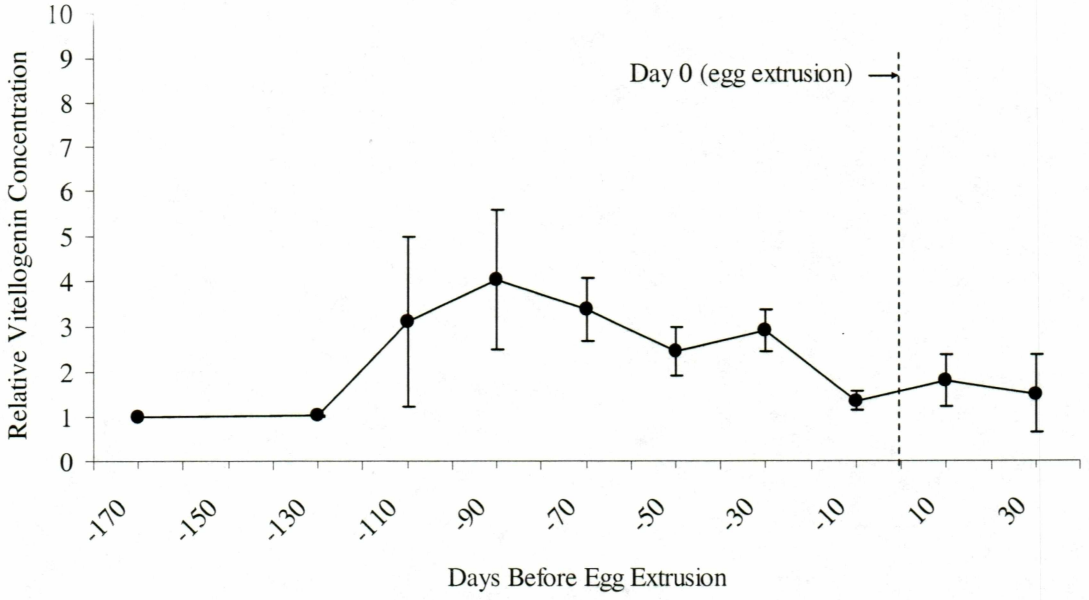


Fig. 3.2. Hemolymph vitellogenin (Vg) concentrations (relative to non-vitellogenic baseline; mean ± SEM) during vitellogenesis in intact female Dungeness crabs. Concentrations decreased from a relative Vg peak (4.0 ± 1.5), 90 days before extrusion to low levels (1.3 ± 0.2), 10 days before egg extrusion.

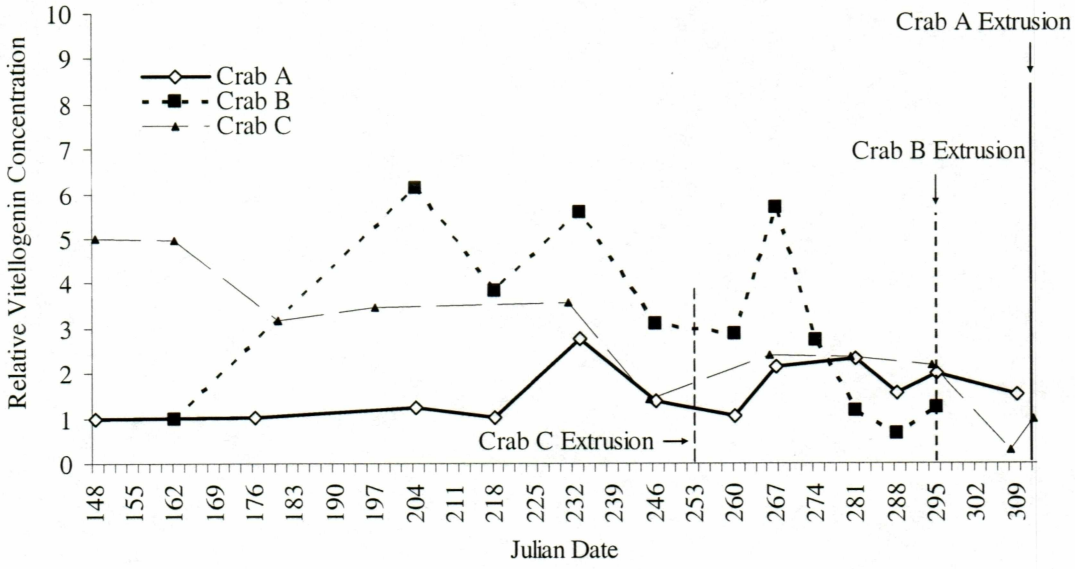


Fig. 3.3. Hemolymph vitellogenin (Vg) levels (relative to non-vitellogenic baseline) during ovarian development in intact Dungeness crabs, maintained in the Juneau laboratory (A and B) and the Seward laboratory (C), plotted against Julian date. Circulating Vg concentrations in crab A and B fluctuated synchronously during vitellogenesis, possibly due to environmental variables. Crab B extruded eggs 18 days prior to Crab A.

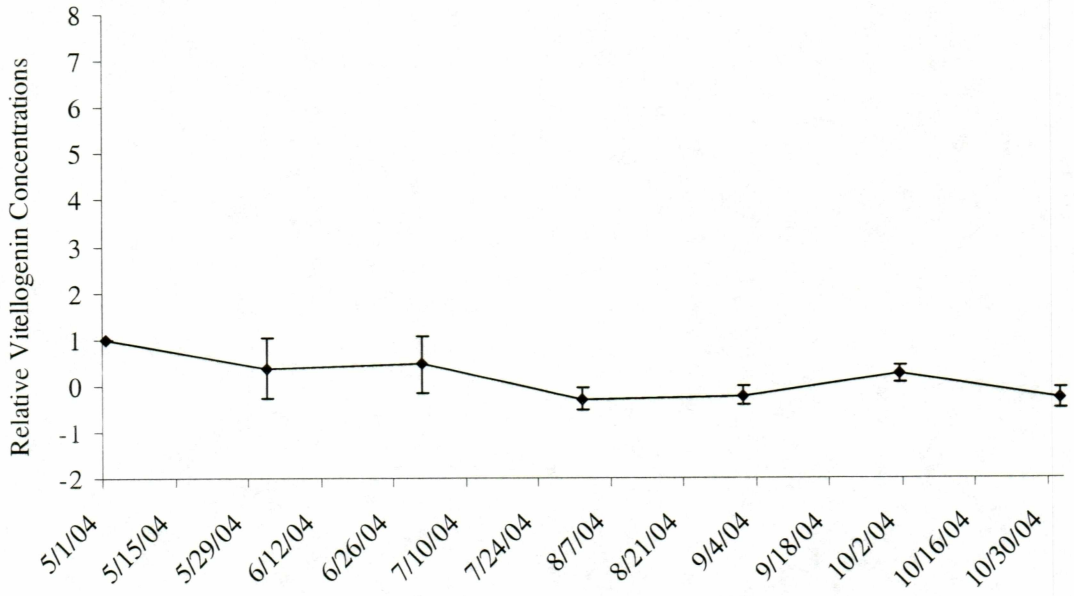


Fig. 3.4. Hemolymph vitellogenin (Vg) levels in non-vitellogenic intact female Dungeness crabs (n=3; mean ± SEM). Hemolymph was sampled during the time of year when ovarian development is observed in most female Alaskan Dungeness crabs.

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CHAPTER 4: CAPTIVE ENVIRONMENTAL CONDITIONS DISRUPT REPRODUCTION AND EFFECT MORTALITY IN DUNGENESS CRAB, CANCER MAGISTER

Abstract: Female Dungeness crabs (*Cancer magister*) (n=73) were collected in southeastern Alaska to facilitate a captive growth and reproductive biology study. The objective was to extract hemolymph monthly from captive female crabs during an annual growth and reproductive cycle. Crabs were maintained in three tanks; monthly hemolymph samples were withdrawn throughout a molting and/or extrusion cycle. Captive crabs began to extrude eggs on 8 September with 29% of the crabs spawning by 9 October, after which all egg extrusion ceased. Coincidently, on 23 September the incoming seawater temperature began to fluctuate between 7.0° and 11.5° C and continued until 9 October. During this time, egg extrusions ceased and significant crab mortality began. Between 23 September and 29 October, 25% of the crabs died; 61% of these crabs had developed ovaries ready for egg extrusion. An unspecified physiologic stress apparently interrupted extrusion and precipitated death. In November, 15% more died, followed by a further 15% in December. When hemolymph samples were analyzed from two dead crabs, no organisms were detected on the direct smear, no growth or anaerobes observed in the culture, and the fungal culture was negative. Tissue analysis revealed no systemic pathogens. Hematopoietic tissues had a high frequency of mitotic

*Prepared for and submitted to the Journal of Zoo and Wildlife Medicine, in review. Thomton, Tuomi, Tamone, and Atkinson, 2006.

figures and pyknotic cells suggestive of abnormal mitoses. Colonies of bacterial rods were also present in autolytic skeletal muscle and clotted hemolymph. Crabs continued to die over the subsequent eight months at a rate of 2% per month. Septicemic shell disease became predominant in the remaining crabs, another common result of physiologic stress. In summary, subsequent to temperature oscillations autotomy of appendages increased, hemolymph became discolored due to egg resorption in the absence of extrusion, and significant mortality occurred, predominantly in the non-ovigerous female group. Ultimately, 52 of the original 73 females died due to bacterial infection, shell necrosis, and physiologic stress during the temperature fluctuations. The onset of these disease conditions was triggered by physiologic stress induced by captive environmental conditions.

Key Words: Cancer magister, Dungeness crab, egg resorption, physiologic stress, shell disease, thermal stress.

INTRODUCTION

Dungeness crab (*Cancer magister* Dana 1852) inhabit coastal waters from Magdalena Bay, Mexico to the Pribilof Islands, Alaska.[7] Annual growth and reproductive cycles vary temporally throughout this range; mating occurs between hard shelled males and newly molted soft shelled females.[3, 9, 16] One currently accepted reproductive cycle of Alaskan female Dungeness crabs maintains that females molt and mate during summer months, extrude eggs in the fall, and incubate eggs through the winter until larval hatching in May and June.[17, 18] In this reproductive strategy, molting and mating are coincident with ovarian maturation. In Alaska, however, an indeterminate but large percentage of females skip the annual molt for at least one year and extrude viable eggs using sperm stored from previous molt/mate events. Additionally, some females may molt and mate in the summer/fall and delay extrusion until the following year. The objective of this study was to quantify circulating ecdysteroid and vitellogenin concentrations in captive female crabs during an annual cycle, in order to assess the molt and reproductive status of the captive crabs.

Two months into the captive experiment, egg extrusion commenced but was abruptly halted and followed by substantial crab mortality. Coincidently, incoming water temperatures widely fluctuated while all other environmental conditions remained stable. These events precipitated a cascade of physiologic stresses and resulted in shell disease and ultimately substantial mortality. This case report presents environmental, physiologic, and histopathologic data related to this event.

CASE REPORT

On 23 July 2003, 73 adult female Dungeness crabs were collected from Port Frederick, Alaska and transported to the Alaska SeaLife Center, in Seward, Alaska to facilitate a growth and reproductive biology study. The female crabs collected for this study were maintained in three separate 2,000 liter flow-through seawater tanks provided with 5 cm of sand substrate. Influent seawater drawn from 100 m depth in Resurrection Bay in the northern Gulf of Alaska was monitored daily for temperature, salinity, dissolved oxygen and pH. Salinity was stable between 31.4 to 33.6 ppt and dissolved oxygen was always greater than 7.0 mg/l. Crabs were fed an assortment of fish, squid and shrimp twice weekly. Throughout a molting and/or extrusion cycle, monthly hemolymph samples were withdrawn from the arthrodial joint at the base of the walking legs using a 26 gauge needle.

Between 23 July and 8 September no molting, egg extrusion or mortality events transpired. The crabs began to extrude eggs on 8 September with 29% of the crabs extruding eggs by 9 October, after which all egg extrusion ceased. Extrusion of eggs in Alaskan Dungeness crabs typically occurs between September and November.[18] Coincidently, on 23 September the incoming seawater temperature began to fluctuate between 7.0° and 11.5° C due to unusual oceanographic conditions in Resurrection Bay and continued until 9 October (Fig. 1). Between 23 September and 12 October, the period of greatest temperature change, the average temperature flux was 1.48° C/24 hours, with a range between 0.78 to 2.56° C/24 hours. Heavily silted supply water

created conditions of zero visibility between 1 - 5 October, and again from 8 - 9 October. During this time, crab extrusions ceased and significant crab mortality began. Between 23 September and 29 October, 25% of the crabs died; 61% of these crabs had fully developed ovaries ready for egg extrusion. It appeared that an unspecified physiologic stress interrupted extrusion and precipitated death.

Due to the heavily silted influent seawater in October the sand substrate solidified and became anaerobic, however hydrogen sulfide was undetectable when measured directly above the sediment. The sediment was removed and tanks cleaned on 28 October. Necropsy of the crab mortalities revealed healthy gills, evidently unaffected by the silt laden supply water.

In November an additional 15% of the crabs died; 91% of these crabs had mature ovaries but failed to extrude, the other 9% had extruded successfully the previous month yet still died. Another 15% died during December; 55% had developed ovaries, 27% had extruded eggs, and 18% had immature ovaries. Following the temperature fluctuations there was an increase in autotomy of appendages, hemolymph was discolored by vitellogenin in the absence of extrusion (due to egg resorption), and significant mortality occurred, predominantly in the non-ovigerous female group.

Hemolymph samples from two crab mortalities were analyzed at the Veterinary Medical Teaching Hospital, University of California, Davis; no organisms were detected on the direct smear, no growth or anaerobes observed in the culture, and the fungal culture was negative. Tissues collected from two freshly dead crabs were analyzed at the Alaska Department of Fish and Game Pathology Laboratory. No systemic pathogens

were reported. Hematopoietic tissues had a high frequency of mitotic figures and pyknotic cells suggestive of abnormal mitoses, and indicative of physiologic stress. Colonies of bacterial rods were also present in autolytic skeletal muscle and clotted hemolymph, most likely due to necrosis of autotomized appendages.

Crabs continued to die in the following eight months at a much slower rate of 2% per month, despite diligent maintenance of the water quality and tank cleanliness. Shell disease caused by abrasive damage and chitinolytic bacteria became predominant on the abdomen and dactyls in the remaining crabs (Fig. 2), which is a common outcome of long-term captivity and chronic physiologic stress.[15] Ultimately, shell disease became systemic and resulted in severely diseased crabs. In summary, 52 of the original 73 females died over 11 months due to physiologic stress induced by captive environmental conditions; the remaining 21 diseased crabs became moribund and were euthanized for necropsy. 16 of these 21 crabs successfully extruded eggs the previous September. The other five crabs did not extrude eggs, however in the subsequent 10 months they exhibited orange hemolymph and bright orange ovaries when dissected.

DISCUSSION

Female Dungeness crabs may extrude eggs based on environmental cues such as photoperiod and water temperature, as well as maturation state of the ovaries. The captive females in this study began to successfully extrude eggs in the fall, however an unidentified factor disrupted this process. The temperature fluctuation that occurred during egg extrusion appears to be the most likely interrupting environmental variable. However, as a frequent inhabitant of the intertidal and shallow sub-tidal zone the thermal

tolerance range of Dungeness crabs is broad. In captivity, Alaskan Dungeness crabs have displayed a 5 to 20° C thermal tolerance range (usually for short exposures),[14] with an increase in mortality only when temperatures exceeded 15° C.[8] Dungeness crabs in the Puget Sound, Washington live in water temperatures ranging from 2.4 to 18.4° C,[4] with lethal temperatures of 32.0° C.[10] In southeastern Alaska, Dungeness crab habitat in lower Glacier Bay was reported to vary annually from 3 to 11° C at 0 m depth, 4 to 9° C at 10 m depth and 4 to 7° C at 50 m depth.[19] Although, the temperature fluctuation observed in this case report is well within the thermal tolerance range for this species over the latitudinal range of the species, Alaskan crabs are acclimatized to colder temperatures. The rate of thermal flux is also similar to conditions in natural Dungeness crab habitat, such as estuaries and shallow bays where tidal flushing and freshwater run-off are dominant forces.

Hypoxic conditions resulting from the presence of anaerobic bottom sediment in the culture tanks may have occurred, however daily dissolved oxygen readings were always greater than 80% (7.0 mg/l). Furthermore, Dungeness crabs are highly tolerant of hypoxic conditions[1] and are commonly found in silty and anaerobic sediment [13].

The physiologic demand of resorbing fully developed ovaries after reproductive disruption accompanied by environmental stress may result in mortality. However, the resorption of eggs by Dungeness crabs has been observed in captivity without a subsequent increase in mortality.[18]

The eventual decline of the surviving crabs from January to July, 2004 was most likely due to systemic chitinolytic bacterial infection, known as shell disease.

Chitinolytic bacteria are common and essential to the breakdown of molted shells from marine crustaceans. Shell disease, also known as black-spot disease, naturally occurs on exoskeletons of living crustaceans, however the molting process usually prevents chronic infection.[20] Severe shell disease has been reported in aquaculture systems due to captive conditions such as overcrowding,[5, 11] and in natural environments due to pollutants.[12, 22] The disease begins with removal of the non-chitinous epicuticle through chemical, physical or biological agents followed by chitinolytic microbial infection.[6] In this study, black-spot lesions were first evident on the abdomen and dactyls, most likely due to abrasive damage from the curved fiberglass tank bottom. The arthrodial membranes at the bases of the walking legs also became infected after repeat perforations with a 26 gauge needle for hemolymph extraction. Eventually the lesions breached the cuticle and resulted in septicemic infection. Shell disease is not normally fatal in the initial stages, but may effect death at later stages of systemic infection of the body cavity, dependent on the nature of the penetrating bacteria.[2, 20, 21]

CONCLUSIONS

The ultimate cause of the significant crab mortality observed remains uncertain. However, the concurrence of egg extrusion and temperature instability may have prevented further oviposition. The subsequent resorption of developed ovaries does not typically result in mortality but may have been interrupted at a critical stage of ovarian development. The physiologic demands imposed by the oscillating water temperature may have exceeded the physiologic tolerance of the crabs at a time when they were already stressed as a result of egg extrusion. The ensuing shell disease epidemic was a

likely outcome of the long-term captive conditions. Recommendations for future captive reproductive crab experiments include: natural sand substrate, aerated sub-sand filtration, and a temperature controlled water supply.

Acknowledgments: This project was supported by University of Alaska Foundation and by NIH Grant Number RR-16466-01 from the RFIP of the National Center for Research Resources. We thank the Alaska SeaLife Center for the use of facilities and the following people for their assistance with crab collections: Jacqueline Mitchell, Chris Siddon, Elizabeth Calvert, Matt Richards and Captain Peter Ord and the crew of F/V Williwaw. We appreciate the laboratory assistance provided Jessica Dutton and Kendall Mashburn. We thank Richard Hocking, Jared Guthridge, Mark Kansteiner, Deanna Trobaugh, and Ed DeCastro at the Alaska SeaLife Center for crab husbandry and hemolymph sample collection. We especially thank Dr. Thomas Shirley for project guidance and editorial support.

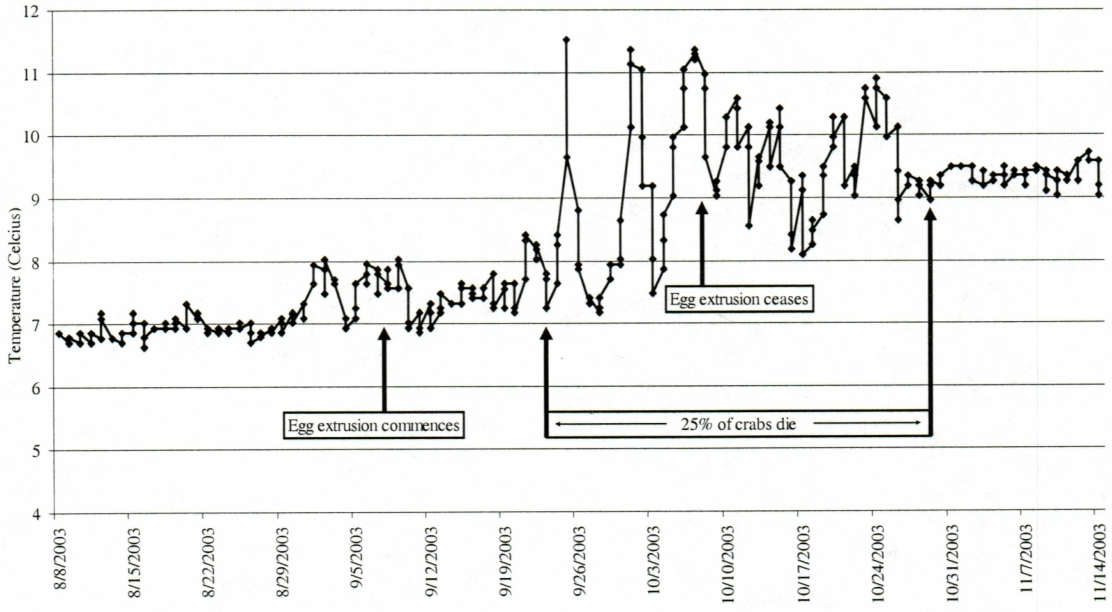

Fig. 4.1. Temperature recordings from captive Dungeness crabs tanks during egg extrusion. Temperatures on 23 September, 2003 increased from 7.3° C to 11.5° C over 40 hours and then continued to widely fluctuate until October 29, 2003. Each symbol represents temperature recording at 8 hour intervals.

Fig. 4.2. A mature female Dungeness crab with severe shell disease on the abdomen, arthrodial joints, and dactyls of the walking legs. Chitinolytic bacterial shell disease is a common result of long-term captivity and physiologic stress and may result in mortality if the disease becomes systemic, as in this specimen.

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CHAPTER 5: GENERAL CONCLUSIONS

In chapter 2, circulating ecdysteroid concentrations were quantified in laboratory and field sampled crabs using an enzyme-linked immunosorbent assay (ELISA), for molt status assessment of individuals. Captive female crabs from southeastern Alaska (mean CW=134.8 mm; n=48) had a typical crustacean molt cycle profile of circulating ecdysteroids. Concentrations of ecdysteroids were low during intermolt (20.3 ± 0.7 ng/ml), maximal during premolt (1,886.5 ± 186.2 ng/ml) 15 days before ecdysis and precipitously declined to low levels (<90 ng/ml) 5 days prior to ecdysis. The duration of premolt was 150 days. Crabs held in captivity either molted (67%), extruded eggs (7%), or demonstrated no reproductive or molting activity (26%). Peak molting occurred in November for crabs held in the laboratory. Most (98%) of the female crabs sampled (n=579) in Port Frederick, Alaska during the expected premolt period (May-July), had intermolt ecdysteroid concentrations. The capability to predict ecdysis 150 days prior to ecdysis by measuring ecdysteroid concentrations provides a useful tool to assess the molt status and timing of ecdysis in Alaskan Dungeness crab populations.

In chapter 3, relative concentrations of circulating vitellogenin (Vg) were quantified during ovarian maturation among induced and non-induced egg extruding crabs. Four of 5 eyestalk ablated (EA) crabs extruded eggs 90 ± 7.4 days after ablation. Vitellogenin concentrations increased approximately 6-fold during ovarian maturation from low levels (1.2 ± 0.3), to peak levels (6.0 ± 1.1) 25 days before egg extrusion, followed by a decrease (2.1 ±0.5), 5 days prior to egg extrusion. Three of 14 intact captive crabs extruded eggs. Circulating Vg increased from low baseline levels (1.0 ± 0.02) 130 days

prior to extrusion, to maximal levels (4.0 ± 1.5) 75 to 100 days before egg extrusion and declined to low6 levels (1.3 ± 0.2) 10 days before egg extrusion. In EA females, vitellogenesis required at least 90 days. In intact females, circulating Vg was detectable ≥100 days before extrusion, enabling the reproductive assessment of individual crabs 3 months prior to extrusion.

Chapter 4 presents a case report following the collection of female Dungeness crabs (*n*=73) collected in southeastern Alaska to. Crabs were maintained in three tanks; monthly hemolymph samples were withdrawn throughout a molting and/or extrusion cycle. Captive crabs began to extrude eggs on 8 September with 29% of the crabs spawning by 9 October, after which all egg extrusion ceased. Coincidently, on 23 September the incoming seawater temperature began to fluctuate between 7.0° and 11.5° C and continued until 9 October. During this time, egg extrusions ceased and significant crab mortality began. Between 23 September and 29 October, 25% of the crabs died; 61% of these crabs had developed ovaries ready for egg extrusion. An unspecified physiologic stress apparently interrupted extrusion and precipitated death. In November, 15% more died, followed by a further 15% in December. When hemolymph samples were analyzed from two dead crabs, no organisms were detected on the direct smear, no growth or anaerobes observed in the culture, and the fungal culture was negative. Tissue analysis revealed no systemic pathogens. Hematopoietic tissues had a high frequency of mitotic figures and pyknotic cells suggestive of abnormal mitoses. Colonies of bacterial rods were also present in autolytic skeletal muscle and clotted hemolymph. Crabs continued to die over the subsequent eight months at a rate of 2% per month. Septicemic

shell disease became predominant in the remaining crabs, another common result of physiologic stress. In summary, subsequent to temperature oscillations autotomy of appendages increased, hemolymph became discolored due to egg resorption in the absence of extrusion, and significant mortality occurred, predominantly in the non-ovigerous female group. Ultimately, 52 of the original 73 females died due to physiologic stress during the temperature fluctuations, bacterial infection and shell necrosis. The onset of these disease conditions was triggered by physiologic stress induced by captive environmental conditions.

The findings of this study further accentuate the complexity and variability of the annual growth and reproductive cycles for Dungeness crabs in Alaskan waters. However, the capability to predict ecdysis 150 days premolt and ovarian development 90 days before egg extrusion provides useful tools for future research of the transition from annual to non-annual growth and reproduction between smaller (100 mm CW) females to the larger (160 mm CW) highly fecund mature female crabs. These techniques could also be useful in assessing molting probabilities in commercially important male Dungeness crabs and other Alaskan crab species.